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# Effects of Ethanol Feeding on Hepatic Lipid Synthesis

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Rats were fed a high-fat, liquid diet containing either 36% of total calories as ethanol or an isocaloric amount of sucrose, for a period up to 35 days. At different time intervals we measured the effects of ethanol administration on the activities of a number of key enzymes involved in hepatic lipid synthesis. At the start of the experimental period the activities of acetyl-CoA carboxylase and fatty acid synthase, measured in liver homogenates, increased in the control as well as in the ethanol-fed group. After 35 days these enzyme activities were still elevated but there were no significant differences between the two groups. In hepatocytes isolated from controls as well as from ethanol-fed rats, short-term incubations with ethanol induced an increase in the rate of fatty acid synthesis and in the activities of acetyl-CoA carboxylase and fatty acid synthase. However, no alterations in the regulation of these enzymes by short-term modulators of lipogenesis were apparent in hepatocytes isolated from alcohol-treated animals. The results do not indicate a major role for the enzymes of *de novo* fatty acid synthesis in the development of the alcoholic fatty liver. The amount of liver triacylglycerols increased in ethanol-fed rats during the entire treatment period, whereas the hepatic levels of phosphatidylcholine and phosphatidylethanolamine were not affected by ethanol ingestion. Ethanol administration for less than 2 weeks increased the activities of phosphatidate phosphohydrolase, diacylglycerol acyltransferase, and microsomal phosphocholine cytidylyltransferase, whereas the cytosolic activity of phosphocholine cytidylyltransferase was slightly decreased. Upon prolonged ethanol administration the activities of these enzymes were slowly restored to control values after 35 days, suggesting development of some kind of adaptation. It is interesting that, although the activities of phosphatidate phosphohydrolase and diacylglycerol acyltransferase were restored to the levels found in the control rats, this effect was not accompanied by a stabilization or decrease of the concentration of hepatic triacylglycerols. 

Chronic administration of ethanol is usually associated with an increased accumulation of hepatic triacylglycerols (TG)[3] which may eventually lead to severe liver damage (reviewed in Refs. (1–3)). These effects have been attributed to an enhancement of the uptake, synthesis, and esterification of fatty acids and particularly to a decrease of their oxidation (1–3). Although enhanced fatty acid synthesis in the liver is considered as one of the mechanisms responsible for the production of ethanol-induced fatty liver, the actual contribution of *de novo* fatty acid synthesis to the hepatic accumulation of fat remains to be settled (1). Acute uptake of ethanol has been found to increase the flux through the pathway of *de novo* synthesis of liver fatty

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[3] Abbreviations used: PMA, 4β-phorbol 12β-myristate 13α-acetate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; VLDL, very low density lipoprotein; TG, triacylglycerol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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acids (1, 4). Prolonged ethanol consumption, on the other hand, was shown to be without effect or to decrease hepatic fatty acid biosynthesis (5, 6). Despite numerous studies concerning the alcoholic fatty liver, knowledge of long-term effects of ethanol feeding on the activity and regulation of enzymes involved in lipid metabolism is scarce. For instance, it is presently unknown whether the enzymes engaged in *de novo* fatty acid synthesis from acetyl-CoA, viz acetyl-CoA carboxylase (EC 6.4.1.2) and fatty acid synthase, play a role in the accumulation of hepatic TG.

Biosynthesis of glycerolipids is regulated by coordinated control of several enzymes. Diacylglycerols are a common precursor for the synthesis of TG and that of the nitrogenous phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The formation of diacylglycerols from phosphatidic acid is catalyzed by the enzyme phosphatidate phosphohydrolase (EC 3.1.3.4). It has been demonstrated that many hormonal and dietary changes in TG synthesis are reflected in changes in the activity of phosphatidate phosphohydrolase (reviewed in Ref. (7)). However, diacylglycerol acyltransferase (EC 2.3.1.20), which catalyzes the conversion of diacylglycerols into TG, is the only enzyme that is exclusively involved in the biosynthesis of TG. There are several indications that diacylglycerol acyltransferase catalyzes an important rate-regulatory step in hepatic TG synthesis (8) and that this enzyme is subject to short-term (8–10), as well as long-term (11) regulation.

Although accumulation of TG upon chronic ethanol administration has been extensively investigated (1–3), much less information is available on the effects of ethanol ingestion on phospholipid biosynthesis in liver. It is generally accepted that CTP:phosphocholine cytidylyltransferase (EC 2.7.7.15) governs the rate of PC synthesis from choline (12), whereas CTP: phosphoethanolamine cytidylyltransferase (EC 2.7.7.14) is the putative rate-limiting enzyme in the corresponding route leading to PE (13).

This paper reports a time study of the effects of ethanol feeding to rats on the activity and regulation of hepatic acetyl-CoA carboxylase and fatty acid synthase, the enzymes responsible for fatty acid synthesis per se. In addition, we followed the changes in the activities of phosphatidate phosphohydrolase and diacylglycerol acyltransferase and tried to correlate these with the alterations in the levels of TG in liver and in serum VLDL. We also compared the effects of prolonged ethanol administration on the activities of the rate-regulatory enzymes of PC and PE biosynthesis with the effects on the levels of these phospholipids in liver. Finally, the effects of ethanol feeding on fatty acid oxidation and on the activity of carnitine palmitoyltransferase have been studied. The results of this study are presented in an accompanying paper (14).

## MATERIALS AND METHODS

*Sources of materials.* [1-$^{14}$C]Acetyl-CoA, [2-$^{14}$C]-malonyl-CoA, [*methyl*-$^{14}$C]phosphocholine, and 1,2-di[1-$^{14}$C]palmitoylphosphatidic acid were obtained from Amersham International (Amersham, UK); $^{3}H_2O$, [1,2-$^{14}$C]ethanolamine, and [1-$^{14}$C]palmitoyl-CoA were purchased from New England Nuclear (Dreieichenhain, FRG). [1,2-$^{14}$C]Phosphoethanolamine was prepared from [1,2-$^{14}$C]ethanolamine according to Sundler (15) using partially purified ethanolamine kinase (16). Bovine serum albumin (fraction V), palmitoyl-CoA, phosphatidylcholine from egg yolk, dipalmitoylphosphatidic acid, and glycerol kinase were from Sigma (St. Louis, MO); ethanol was provided by Nedalco (Bergen op Zoom, The Netherlands); insulin and glucagon were kindly donated by Lilly Research Laboratories (Indianapolis, IN). The origin of other chemicals has been described previously (17).

*Animals and their treatment.* Male Wistar rats (195 ± 8 g initial body weight) were used throughout this study. They were divided in two groups; alcohol-fed and control. Both groups were fed the DeCarli-Lieber diet, which in essence is a high-fat liquid diet (18). The alcohol-treated group received 36% of total calories as ethanol, 35% as fat (mostly unsaturated), 18% as protein (supplemented with cysteine and methionine), and 11% as carbohydrate. In the control group, ethanol was replaced by an isocaloric amount of sucrose. The animals were kept in a constant-temperature room with a 12 h light (6 AM–6 PM)–dark (6 PM–6 AM) cycle up to 35 days. The average intake of food of the ethanol-fed animals was not significantly different from that of the controls. At the end of a 5-week dietary treatment period the average body weight was 295 ± 10 g for the alcohol-fed group and 305 ± 15

g for the control group (difference not significant by the paired *t* test). A group of rats fed a standard, low-fat pelleted diet was considered to be the zero-time control of the treatment.

*Preparation of biological samples.* Animals were sacrificed by decapitation at 9 AM. Blood was collected in centrifuge tubes for serum isolation and the livers were excised. Four pieces of each liver were homogenized (25% homogenate, w/v) on ice for subsequent enzyme assays. The piece for measurement of acetyl-CoA carboxylase activity was homogenized with a loose-fitting Dounce homogenizer (five strokes) in a medium containing 50 mM Hepes (pH 7.5), 0.25 M mannitol, 4.0 mM citrate, 6.16 mM EDTA, and 5.0 mM 2-mercaptoethanol. An additional volume of a citrate/EDTA solution was added to bring these compounds to final concentrations of 4.0 and 6.16 mM, respectively.

The crude homogenate was centrifuged at 12,000*g* for 5 min and the resulting supernatant was used for enzyme assays. The activity of lactate dehydrogenase was measured in 12,000*g* supernatant (19) and compared to the total activity as measured in the presence of Triton X-100 (20). Consequently, the recovery of cytosolic protein in the homogenate could be assessed.

The piece of liver intended for the measurement of fatty acid synthase was homogenized in a buffer containing 10 mM Hepes (pH 7.5) and 0.25 M sucrose in the way described above for the carboxylase. Following 5 min spinning, the 12,000*g* supernatant was used for determining the activity of fatty acid synthase. The activity of lactate dehydrogenase was also measured in this supernatant. For measuring the activities of diacylglycerol acyltransferase and CTP:phosphocholine and CTP:phosphoethanolamine cytidylyltransferases, part of the liver was homogenized with a glass-Teflon Potter-Elvehjem homogenizer in 4 vol of a buffer containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10 mM NaF. For determination of the activity of phosphatidate phosphohydrolase the homogenate was prepared in a buffer containing 0.25 M sucrose and 10 mM Hepes (pH 7.4) using the Potter-Elvehjem homogenizer. The homogenates were centrifuged at 12,000*g* for 15 min, followed by centrifugation of the supernatant at 105,000*g* for 60 min. The supernatant of the latter centrifugation step was considered as the cytosolic fraction. The microsomal pellets were resuspended in their respective homogenization buffers to a protein concentration of 5 mg/ml.

*Isolation and incubation of hepatocytes.* Hepatocytes were isolated and purified essentially according to the procedure of Seglen (21), as described previously (22). Isolated cells were suspended in Krebs-bicarbonate buffer supplemented with 10 mM glucose and 1% (w/v) defatted and dialyzed bovine serum albumin.

Incubations were carried out in a total volume of 1 ml in a gyratory metabolic shaker (85 revolutions/min) at 37°C in 25-ml Erlenmeyer flasks which contained about 3.5–5 mg of cellular protein/ml. During incubation the flasks were continuously gassed with 95% oxygen and 5% carbon dioxide. Enzyme assays were performed on cells incubated for 30 min. For measuring the rate of fatty acid synthesis cells were incubated for 60 min.

*Measurement of acetyl-CoA carboxylase activity.* The activity of acetyl-CoA carboxylase was measured by determining the incorporation of [1-$^{14}$C]acetyl-CoA into fatty acids in a reaction coupled to the fatty acid synthase reaction. Diluted 12,000*g* supernatant (0.55–0.75 mg protein) was prewarmed for 30 min at 37°C. Assays were started by the addition of 100 μl preheated assay mixture (37°C) to 100 μl of the biological samples. Reactions were stopped after 1 min by addition of 100 μl 10 M NaOH. The total incubation mixture for the assay contained 78 mM Hepes (pH 7.5), 125 mM mannitol, 2.5 mM 2-mercaptoethanol, 3.1 mM EDTA, 4.0 mM citrate, 5.6 mM $MgCl_2$, 2.0 mM ATP, 20 mM $KHCO_3$, 0.93% (w/v) bovine serum albumin (charcoal treated and dialyzed), 0.44 mM dithioerythritol, 0.5 mM NADPH, 0.062 mM butyryl-CoA, 0.062 mM [1-$^{14}$C]acetyl-CoA (4 Ci/mol), and 3.2 mU fatty acid synthase. The rest of this procedure was performed exactly as described in (17).

Acetyl-CoA carboxylase activity in isolated hepatocytes was determined by a coupled assay in digitonin-permeabilized cells exactly as described before (17).

*Measurement of fatty acid synthase activity.* The activity of fatty acid synthase in homogenates was determined by measuring the incorporation of [1-$^{14}$C]-acetyl-CoA into fatty acids in the presence of malonyl-CoA. One hundred microliters diluted 12,000*g* supernatant (0.40–0.60 mg protein) was prewarmed for 5 min at 37°C. The reactions were started by the addition of 100 μl preheated assay mixture (37°C). Assays were stopped after 5 min by adding 100 μl 10 M NaOH. The total incubation mixture contained 100 mM Hepes (pH 6.8), 125 mM sucrose, 0.5 mM NADPH, 0.44 mM dithioerythritol, 1 mM EDTA, 0.93% (w/v) bovine serum albumin (charcoal treated and dialyzed), 0.195 mM malonyl-CoA, and 0.062 mM [1-$^{14}$C]-acetyl-CoA (4 Ci/mol). Fatty acids were extracted and quantified as described above.

Assessment of fatty acid synthase activity in isolated hepatocytes was carried out in digitonin-permeabilized cells exactly as described earlier (17).

*Other enzyme assays.* CTP:phosphocholine cytidylyltransferase activity was measured in the microsomal and in the cytosolic fractions as described by Pelech *et al.* (23). The cytosolic activity was determined in the absence or presence of 500 μg total liver phospholipid (24). The total activity of CTP:phosphoethanolamine cytidylyltransferase was assayed in the cytosol by measurement of the rate of conver-

sion of [1,2-$^{14}$C]phosphoethanolamine into CDP-[1,2-$^{14}$C]ethanolamine (25).

The activity of diacylglycerol acyltransferase was assayed in the microsomal pellet by measuring the incorporation of [1-$^{14}$C]palmitoyl-CoA into TG using endogenous diacylglycerols as the second substrate (10). The activity of phosphatidate phosphohydrolase was determined in the 105,000*g* supernatant as well as in the microsomes by measuring the production of [$^{14}$C]diacylglycerols from 1,2-di[$^{14}$C]palmitoylphosphatidic acid as described by Martin *et al.* (26). The incubations were performed with 60–90 μg cytosolic protein or with 80–120 μg microsomal protein for 30 min at 37°C.

*Further analytical procedures.* Fatty acid synthase—utilized in the carboxylase assays—was isolated and purified from rat liver according to Linn (27), and stored at −80°C. The donor animals for this isolation were starved 72 h and then refed a fat-free, high-sucrose diet for 48 h.

Liver lipids were extracted according to Bligh and Dyer (28). Separation and quantification of TG, PC, and PE were carried out as described previously (25). Sera of three rats were combined and serum lipoproteins were separated according to Terpstra (29). TG concentrations in VLDL were determined with an enzymatic kit supplied by Boehringer (Mannheim, FRG) using a multistat III Plus F/LS (Instrumentation Laboratory).

Free glycerol levels in deproteinized serum samples were analyzed using glycerol kinase and glycerol-3-phosphate dehydrogenase (30).

To monitor rates of *de novo* fatty acid synthesis in hepatocytes $^{3}H_2O$ (0.5 Ci/ml) was added to the cellular incubations. Quantification of lipogenesis was performed as published earlier (17). In isolated hepatocytes absolute rates of fatty acid synthesis vary from cell preparation to cell preparation (31). As the short-term effects of agonists, expressed as percentage of control, are reproducible between experiments, the data from cell experiments are expressed as percentage of control.

Protein was determined according to Lowry *et al.* (32) using bovine serum albumin as standard. All enzyme assays were performed in duplicate. Results are presented as the means ± SD of three identically treated animals. Statistical analysis was performed using the paired *t* test.

## RESULTS

To minimize interference of the carboxylase assay by mitochondrial enzymes, breakage of mitochondria was limited by homogenizing the pieces of liver with a loose-fitting, all glass Dounce homogenizer. Mitochondria were subsequently removed from the homogenates by centrifugation. Including purified fatty acid synthase in the assay couples the carboxylation to the fatty acid synthase reaction. In this way, malonyl-CoA will not accumulate to levels which inhibit carboxylase activity and significant decarboxylation of malonyl-CoA by fatty acid synthase will be prevented (cf. Ref. (17)).

Preliminary experiments had shown that the coupled acetyl-CoA carboxylase assay in 12,000*g* supernatants proceeded at linear rates up to at least 1.5 min with 0.25–1.25 mg protein in the incubation. The assay performed to determine the activity of fatty acid synthase in 12,000*g* supernatants was linear for at least 6 min with 0.25–0.75 mg protein in the assay.

In all experiments the recovery of lactate dehydrogenase in 12,000*g* supernatants was higher than 95% of that released with Triton X-100.

*Profile of the activities of acetyl-CoA carboxylase and fatty acid synthase during the experimental period.* The effects of ethanol consumption on the enzymes concerned with *de novo* fatty acid synthesis were investigated in rats fed a high-fat diet containing 36% of total calories as ethanol (alcohol-fed rats) or an isocaloric amount of sucrose (control rats) for 35 days. During this period the alcohol-fed rats accumulated substantially more TG in their liver than control animals (Fig. 1A). The activities of acetyl-CoA carboxylase and fatty acid synthase were measured at different time points in the course of the 5-week experimental period.

As can be inferred from Figs. 2A and 2B, the profiles of the activities of acetyl-CoA carboxylase and fatty acid synthase during the dietary treatment period followed a qualitatively similar trend: in the control group both enzyme activities rapidly increased. In the ethanol-fed animals these enzyme activities also increased but the response was delayed as compared to that of control rats. This delay was more pronounced for the carboxylase than for the synthase. The magnitude of the elevation in activity in the control and treated group was similar for the two enzymes. It is noteworthy that at the end of the experimental period the enzyme activities were still elevated but there were no significant differ-

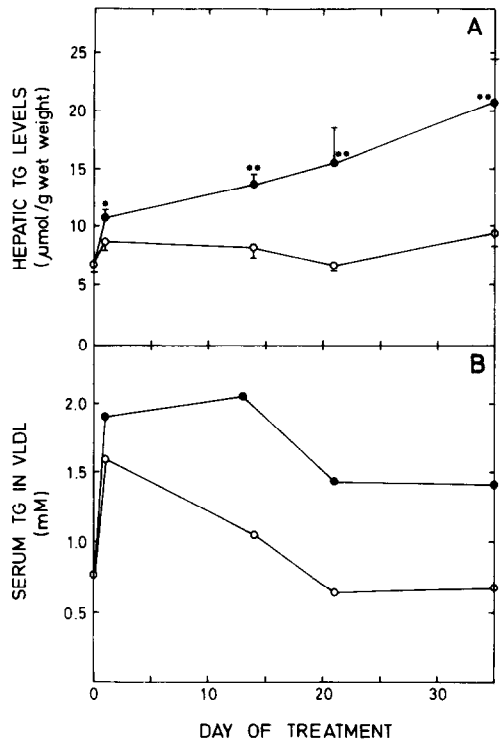


FIG. 1. Effects of ethanol feeding on hepatic and serum levels of triacylglycerols (TG). TG were isolated from liver homogenates (A) or from serum VLDL (B) in the course of a 35-day experimental period from ethanol-treated (●) or control (○) animals. The sera of three identically treated rats were pooled and triplicate determinations of TG were performed per pool at each time point. Significance of difference from control: $*P < 0.05$; $**P < 0.01$.

ences in the activities of the two enzymes between ethanol-treated animals and controls.

Acute ethanol administration has been reported to enhance fatty acid release from adipose tissue (1). Alcohol-triggered lipolysis in adipose tissue may contribute to the delay observed in the increase in activity of acetyl-CoA carboxylase in the first 2 weeks of alcohol feeding (Fig. 2A). Therefore, the levels of serum free glycerol were determined throughout the dietary treatment period. Surprisingly, the serum concentration of free glycerol was not higher in the ethanol-fed rats than in the controls during the first period of the treatment (data not shown). Hence, no direct relation seems to exist between lipolytic processes in adipose tissue and the delay in the increase of the activity of liver acetyl-CoA carboxylase in the initial stage of alcohol ingestion.

*Studies with isolated hepatocytes.* The disappearance of the initial differences in enzyme activities between the two groups points to adaptive changes in the regulatory properties of these enzymes. Therefore, experiments were initiated to study short-term regulation of fatty acid synthesis, acetyl-CoA carboxylase, and fatty acid synthase in hepatocytes isolated from alcohol-fed and control rats after 35 days of dietary treatment.

Table I shows the short-term responses of acetyl-CoA carboxylase to ethanol, hormones (insulin, glucagon), and phorbol myristate acetate (PMA). Exposure of hepatocytes to ethanol induced an increase in the activity of acetyl-CoA carboxylase in both "control cells" and "alcohol cells." This stimulatory effect of ethanol on the activity of acetyl-CoA carboxylase in the two cell types (Table I) was also observed on the rate of fatty acid synthesis in those

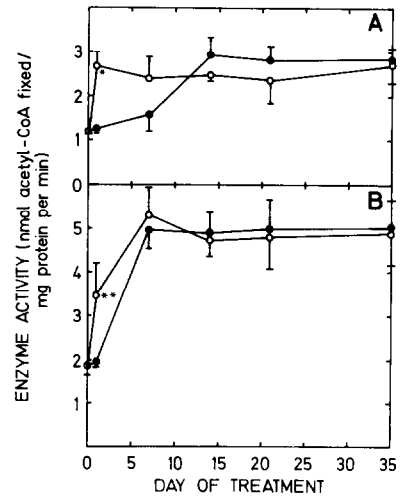


FIG. 2. Profiles of the activities of acetyl-CoA carboxylase and fatty acid synthase in liver in the course of a 5-week dietary treatment period. Ethanol-treated (●) and control rats (○). (A) Acetyl-CoA carboxylase activity; (B) fatty acid synthase activity. The data are expressed in nmol/mg 12,000*g* supernatant protein per minute. Assays were carried out in triplicate per animal. Significantly different from control: $*P < 0.01$; $**P < 0.02$.

TABLE I

SHORT-TERM EFFECTS OF ETHANOL, HORMONES, AND PMA ON THE ACTIVITY OF ACETYL-CoA CARBOXYLASE IN ISOLATED HEPATOCYTES

| Addition | Carboxylase activity (%) | |
|---|---|---|
| | Control group | Alcohol group |
| None | 100 | 100 |
| Ethanol, 20 mM | 119 ± 10 | 146 ± 11 |
| Insulin, 85 nM | 138 ± 5 | 136 ± 14 |
| Glucagon, 10 nM | 74 ± 12 | 91 ± 11 |
| PMA, 100 nM | 115 ± 8 | 121 ± 19 |
| Ethanol + insulin | 157 ± 15 | 205 ± 24 |
| Ethanol + glucagon | 86 ± 11 | 104 ± 11 |
| Ethanol + PMA | 134 ± 10 | 181 ± 21 |

*Note.* Cells were isolated after 35 days in the experimental period. The data are expressed as percentage of incubations without additions. Values represent the means ± SD for three animals of each dietary group with three independent incubations per cell preparation. The 100% levels were 1.11, 0.80, and 1.16 nmol acetyl-CoA fixed/mg cellular protein per minute for the three control experiments and 1.17, 0.74, and 0.46 for the three cell preparations from ethanol-fed animals.

cells (Fig. 3A). The ethanol effect on fatty acid synthesis and on acetyl-CoA carboxylase activity depended on the magnitude of the basal rate of this process, viz with low rates the percentage stimulation was higher than with high rates. Hepatic acetyl-CoA carboxylase activity is enhanced only following incubation of cells with ethanol. The presence of this drug during enzyme assay did not result in a change in carboxylase activity (Table II).

The addition of insulin, glucagon, or PMA produced the expected effects on acetyl-CoA carboxylase activity in control cells. Ethanol treatment of animals for 35 days did not change the response of hepatocellular acetyl-CoA carboxylase to these short-term modulators of metabolism (Table I).

Incubation of hepatocytes in the presence of ethanol unexpectedly increased fatty acid synthase activity as measured in digitonin-permeabilized hepatocytes (Fig. 3B). This small but very reproducible effect of ethanol on hepatocellular fatty acid synthase activity was noted in both "control cells" and "alcohol cells." The presence of ethanol only during the assay failed to affect fatty acid synthase activity (Table II). The short-term stimulation of fatty acid synthase activity by ethanol is still observed when [1-$^{14}$C]acetyl-CoA in the enzyme assay is substituted for [2-$^{14}$C]-malonyl-CoA (Table II). This excludes the possibility that the enhanced activity of fatty acid synthase is an artifact which is caused by an increased [1-$^{14}$C]acetyl-CoA–malonyl-CoA label exchange reaction.

*Effects of ethanol feeding on TG synthesis.* During the experimental period the amount of hepatic TG in ethanol-fed rats quickly increased (Fig. 1A). Moreover, in accordance with earlier studies (1-3), the level of TG in serum VLDL was rapidly enhanced in ethanol-treated animals as compared to controls (Fig. 1B).

Phosphatidate phosphohydrolase and diacylglycerol acyltransferase may both

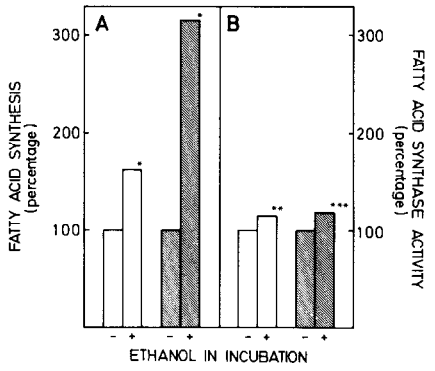


FIG. 3. Rates of fatty acid synthesis (A) and activities of fatty acid synthase (B) in hepatocytes isolated after 35 days in the experimental period. The data are expressed as percentage of incubations without additions. Values represent the means ± SD for three animals of each dietary group with three independent incubations per cell preparation. "Alcohol cells," shaded bars; "control cells," open bars; incubations with 20 mM ethanol (+) or without (−). The 100% level for the rate of lipogenesis is 46 (75, 34, 29) and 133 (184, 59, 156) nmol acetyl equivalents/mg cell protein per hour for alcohol cells and control cells, respectively. The corresponding 100% level for the activity of fatty acid synthase is 4.2 (4.9, 3.6, 4.1) and 4.3 (4.6, 5.0, 3.4) nmol acetyl-CoA fixed/mg cell protein per minute, respectively. Significantly different from control: $^{*}P < 0.01$; $^{**}P < 0.025$; $^{***}P < 0.05$.

TABLE II

EFFECTS OF THE PRESENCE OF ETHANOL IN ENZYME ASSAYS OR IN CELL INCUBATIONS ON ACETYL-CoA CARBOXYLASE AND FATTY ACID SYNTHASE ACTIVITIES

| Presence of ethanol | | Acetyl-CoA carboxylase (%) | Fatty acid synthase (%) |
|---|---|---|---|
| Assay | Cell incubation | | |
| − | − | 100 | 100 |
| + | − | 100 ± 4 | 100 ± 4 |
| + | + | 150 ± 7 | 130 ± 4[a]* |
| + | + | — | 139 ± 7[b]* |

*Note.* Cells were isolated from rats fed the standard low-fat pelleted diet. The data are expressed as percentage of incubations without additions. Concentration of ethanol in incubations, 20 mM; in assays, 10 mM. Significantly different from control: $*P < 0.01$.

[a] Activity measured by incorporation of [1-$^{14}$C]acetyl-CoA into fatty acids.

[b] Activity measured by incorporation of [2-$^{14}$C]malonyl-CoA into fatty acids.

play an important role in controlling the rate of TG synthesis (7–9). Figures 4A and 4B demonstrate that ethanol ingestion induced an increase of the activity of phosphatidate phosphohydrolase. The stimulation of the cytosolic activity was maximal between Day 7 and Day 14. After 2 weeks of ethanol feeding this phosphohydrolase activity was gradually restored to the level found in control animals (Fig. 4A). The increase of the microsomal phosphatidate phosphohydrolase activity was faster than that of the cytosolic enzyme (Fig. 4B). After 1 day of treatment the activity in the ethanol-treated animals was almost double that in control rats. The activity in the ethanol-fed rats continued to increase until Day 14 (Fig. 4B) and from then on seemed to decrease. After 35 days of ethanol feeding the microsomal phosphatidate phosphohydrolase activity was no longer significantly different from that measured in the control group.

Figure 4C demonstrates that the activity of diacylglycerol acyltransferase also considerably increased upon ethanol ingestion. After 14 days the hepatic activity of diacylglycerol acyltransferase in ethanol-treated rats was more than twice that in livers of control animals. Prolonged treatment of rats with ethanol resulted in a steady decrease of diacylglycerol acyltransferase activity and after 35 days the enzyme activity in ethanol-fed animals was no longer significantly different from that of the controls.

*Effects of ethanol feeding on phospholipid biosynthesis.* The hepatic levels of PC and PE of alcohol-fed animals were not significantly different from those of the control group (not shown).

It is generally agreed that CTP:phosphocholine cytidylyltransferase plays an important role in the determination of the rate of PC synthesis (12). Ethanol administration to rats induced an immediate increase of the microsomal phosphocholine cytidylyltransferase activity (Fig. 5A). During the following 2 weeks the microsomal cytidylyltransferase activity of the

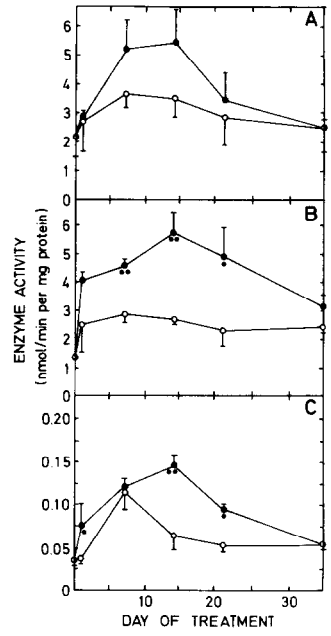


FIG. 4. Effects of ethanol treatment on the activities of phosphatidate phosphohydrolase and diacylglycerol acyltransferase. Enzyme activities were determined in the course of a 5-week dietary treatment period in ethanol-treated (●) or control rats (○). (A) Phosphatidate phosphohydrolase activity in cytosol; (B) phosphatidate phosphohydrolase activity in microsomes; (C) diacylglycerol acyltransferase activity in microsomes. Significance of difference from control: $*P < 0.05$; $**P < 0.01$.

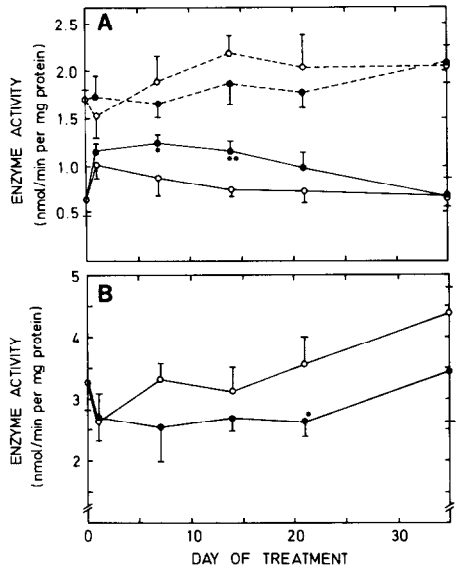


FIG. 5. Effects of ethanol feeding on the activities of CTP:phosphocholine cytidylyltransferase and CTP:phosphoethanolamine cytidylyltransferase in the course of a 5-week dietary period. Enzyme activities were determined in livers of ethanol-fed (●) or control rats (○) at each time point. (A) phosphocholine cytidylyltransferase activity in microsomes (solid lines) and in cytosol (dashed lines). The cytosolic activity was determined in the presence of total liver phospholipid; (B) phosphoethanolamine cytidylyltransferase activity in cytosol. Significance of difference from control: $*P < 0.05$; $**P < 0.01$.

alcohol-fed animals was significantly higher than that of the controls. After ethanol feeding for 35 days, the enzyme activity was equal to that found in livers of control animals. While the microsomal enzyme activity was increased by ethanol treatment, the activity of the cytosolic phosphocholine cytidylyltransferase activity, determined in the presence of phospholipids, was slightly lower in ethanol-treated animals than in controls, although these differences were not statistically significant (Fig. 5A). The activity of cytosolic phosphocholine cytidylyltransferase, assayed in the absence of phospholipids, was not affected by ethanol ingestion (results not shown).

Figure 5B demonstrates that the effects of ethanol consumption on the activity of CTP:phosphoethanolamine cytidylyltransferase were quite different from those on the activity of phosphocholine cytidylyltransferase (cf. Figs. 5A and 5B). Whereas phosphocholine cytidylyltransferase activity seemed to be enhanced by ethanol treatment, the activity of phosphoethanolamine cytidylyltransferase was slightly lower in ethanol-treated animals than in controls (Fig. 5B). Figure 5B demonstrates that the activity of phosphoethanolamine cytidylyltransferase rapidly decreased in control as well as in alcohol-fed animals upon feeding a high-fat diet, but that the enzyme activity in control animals was restored faster than in ethanol-treated rats.

## DISCUSSION

In the present study and in a following paper (14) the effects of alcohol treatment on a great number of lipid metabolic parameters is determined within a single experimental format. The results presented cover both acute and chronic outcomes of ethanol administration and may clarify some of the disparate results in the literature. Overall, the goal is to explain the accumulation of TG in the alcoholic fatty liver.

We determined the time course of the effects of ethanol consumption on the activity of a number of key enzymes of hepatic lipid synthesis, viz acetyl-CoA carboxylase, fatty acid synthase, phosphatidate phosphohydrolase, diacylglycerol acyltransferase, CTP:phosphocholine cytidylyltransferase, and CTP:phosphoethanolamine cytidylyltransferase. Although there are several reports on the acute or chronic effects of ethanol feeding on some of these enzyme activities (33–37), this is the first study presenting a detailed time course of the effects of ethanol consumption on the activity of the aforementioned enzymes in rat liver.

From the results presented in Fig. 2 it is obvious that the test diet induces an increase of the hepatic activities of acetyl-CoA carboxylase and fatty acid synthase when compared to the zero time controls, i.e., the rats fed a standard, low-fat pelleted diet. Alcohol administration delayed this enhancement to some extent. Apart from ethanol, the dietary treatment of the

control group and the alcohol-fed group differs in the amount and type of carbohydrate. The basic test diet—offered to both groups of animals—contains carbohydrate. In the dietary regime of the control rats sucrose was substituted for ethanol. This protocol is generally used for the study of ethanol effects. However, the rise in the activities of the two lipogenic enzymes, which was first observed in the control group, may well be evoked by the consumption of the readily digestible carbohydrate. High-sucrose diets are known to cause induction of lipogenic enzymes *in vivo* (38). Furthermore, both glucose (39) and fructose (40) have been shown to induce lipogenic enzymes in isolated hepatocytes. The increase in the enzyme activities in the alcohol-fed group (Fig. 2) suggests the presence of such an inductor for lipogenic enzymes in these animals as well. The nature of this inductor is, as yet, unknown.

In the first 2 weeks of the experimental period, the increase in activity of acetyl-CoA carboxylase in the alcohol-fed group was delayed as compared to the controls. As the serum glycerol concentration did not change in ethanol-treated rats, this delay did not result from an increased supply to the liver of fatty acids—known inhibitors of lipogenesis (41)—derived from enhanced lipolysis in adipose tissue. It is possible, on the other hand, that the delay in carboxylase activity in the alcohol-fed group in the first 2 weeks of the experimental period has been induced by fatty acids from intestinal origin rather than from adipose tissue. Acute administration of ethanol has been shown to increase the intestinal lymph flow and output of dietary lipids (42). However, the stimulatory effect of ethanol on output of lipids by the lymph ceases after several weeks of alcohol consumption (42). This would be in line with our observation that the alcohol-determined delay in carboxylase activity disappears after 2 weeks of alcohol ingestion (Fig. 2). Alternatively, the increased FABP content in livers of alcohol-fed rats (43) enlarges the capacity for binding fatty acids and may thereby eliminate an effect of fatty acids on the carboxylase.

Possible alterations in the regulation of lipogenesis resulting from prolonged alcohol consumption were studied with hepatocytes isolated at the end of the 35-day experimental period. In these hepatocytes short-term regulation of the rate of fatty acid synthesis and of the activities of acetyl-CoA carboxylase and fatty acid synthase by alcohol and certain agonists have been studied. The stimulation of fatty acid synthesis noted *in vivo* upon acute administration of ethanol (40) is also observed in isolated hepatocytes exposed to acute doses of ethanol. The effect is most pronounced in "alcohol cells" (Fig. 3A). The ethanol-induced stimulation of fatty acid synthesis has been suggested to be mainly due to the increased hepatic oxidation of ethanol yielding reducing equivalents (44). This explanation, however, is not very likely. It is generally accepted that the capacity for NADPH generation is not limiting lipogenesis since the activities of the enzymes responsible for NADPH production are at least one order of magnitude higher than those of the enzymes involved in fatty acid synthesis per se irrespective of nutritional or hormonal state (45–47).

The absolute rates of fatty acid synthesis in isolated hepatocytes vary significantly from experiment to experiment (cf. legend to Fig. 3). Therefore, it is quite difficult to draw conclusions from *in vitro* experiments—as done by others (cf. Ref. (6))—as to the actual effect of chronic ethanol consumption on the rate of hepatic fatty acid synthesis. *In vivo* experiments seem to be more appropriate for that purpose.

The regulatory properties of acetyl-CoA carboxylase in "alcohol cells" following short-term exposure of isolated liver cells to certain agonists did not change (Table I).

The short-term stimulatory effect of ethanol on the activity of fatty acid synthase both in control cells and in alcohol cells (Table II, Fig. 3B) has not been observed before. It is the digitonin-permeabilized cell assay (17) which rapidly measures enzyme activity without preparing subcellular fractions that allowed us to observe this phenomenon. Other short-term modulators of cellular lipid metabolism

like hormones failed to affect the activity of this enzyme (17) indicating a very specific effect of ethanol. The cellular activity of acetyl-CoA carboxylase—as measured in the permeabilized-cell assay—is also enhanced by ethanol (Table I). For the activation of the carboxylase and the synthase metabolism of ethanol seems to be a prerequisite. The presence of ethanol only during assay of the enzymes failed to affect their activity. The observation that the carboxylase as well as the synthase are responsive to short-term treatment of cells with ethanol is interesting. The increased sensitivity of these lipogenic enzymes to acute doses of ethanol may help to sustain the hepatic accumulation of TG during chronic alcohol consumption.

While the effects of ethanol ingestion on TG synthesis have been extensively investigated (1–3), much less information is available in this connection about enzymes involved in phospholipid synthesis. The results of various reports on the effects of ethanol administration on liver phospholipid levels are quite contradictory. Several studies demonstrated an accumulation of phospholipids upon prolonged ethanol administration to rats (48, 49), whereas others reported that chronic ethanol consumption did not affect hepatic phospholipid levels in rats and in other species (36, 50, 51). The amounts of hepatic PC and PE in ethanol-treated rats were not significantly different from the levels found in control animals during a treatment period of 35 days.

Although the effects of ethanol consumption on the amounts of hepatic PC and PE are more or less comparable, the effects of the diet on the activities of the key regulatory enzymes of PC and PE synthesis, phosphocholine and phosphoethanolamine cytidylyltransferases, were completely different (Fig. 5). Ethanol treatment slightly decreased the activity of phosphoethanolamine cytidylyltransferase. However, this did not result in a decrease of the hepatic PE concentration. This may indicate that either the turnover of PE has been changed by ethanol treatment or that other, yet unknown, factors may play a role in determining the rate of PE synthesis.

There is accruing evidence that the active form of phosphocholine cytidylyltransferase is located in the endoplasmic reticulum, whereas the enzyme present in the cytosol serves as a reservoir (12). Several studies have indicated that consumption of ethanol induces an increase of the amount of hepatic fatty acids (reviewed in (1, 3)). Pelech *et al.* (52) reported that incubation of HeLa cells in the presence of fatty acids resulted in enhanced PC synthesis. This effect was accompanied by a translocation of phosphocholine cytidylyltransferase from the cytosolic to the microsomal compartment. The results of our study clearly indicate that ethanol ingestion results in an enhanced activity of the microsomal form of phosphocholine cytidylyltransferase (Fig. 5A). Concomitantly, the activity of the cytosolic enzyme, measured with phospholipids, is slightly lower in alcohol-fed rats than in control animals, although the latter effect was not statistically significant. Thus, the increase of microsomal enzyme activity may be attributed to a translocation of the enzyme from the cytosol to the endoplasmic reticulum, possibly as a consequence of the enhanced concentration of hepatic fatty acids in ethanol-treated animals. However, activation of phosphocholine cytidylyltransferase did not result in an increase of hepatic PC concentration (not shown). Our observations imply that either phosphocholine cytidylyltransferase activity is not rate limiting under these conditions, or, more likely, that the turnover of PC is enhanced. Such enhanced turnover has indeed been demonstrated in liver microsomes from rats chronically fed ethanol (49). In addition, it has been reported that the formation of PC by methylation of PE was also stimulated in livers of ethanol-treated rats (50).

Figure 1A demonstrates that ethanol ingestion is followed by hepatic TG accumulation. While the activities of phosphatidate phosphohydrolase and diacylglycerol acyltransferase were restored to control levels after 35 days of ethanol treatment (Fig. 4), the concentration of liver TG continued to increase during this period. In the following paper we report that fatty acid oxidation was inhibited in hepato-

cytes isolated from ethanol-fed rats as compared to controls (14). It is likely that this inhibition is one of the origins of the continued increase of hepatic TG, despite a normalization of the activities of phosphatidate phosphohydrolase and diacylglycerol acyltransferase as shown in Fig. 4.

The formation of TG from diacylglycerol and acyl-CoA is determined by the activity of diacylglycerol acyltransferase. Previously, the activity of this enzyme had been reported to be unaltered upon short-term ethanol treatment (34), although others observed a threefold increase after acute ethanol administration when rat liver microsomes were incubated in the presence of cytosol (37). Haagsman and Van Golde (53) demonstrated that the effects of exogenous fatty acids on cellular diacylglycerol acyltransferase activity paralleled those on TG synthesis and VLDL secretion in isolated hepatocytes. Our results demonstrate that in the initial stage of ethanol ingestion both diacylglycerol acyltransferase activity and VLDL secretion increased. However, although the enzyme activity was restored to control levels after 35 days of ethanol consumption (Fig. 4C), the concentration of TG in serum VLDL in alcohol-fed rats was still considerably higher at that time as compared to the control rats. Our data are comparable to those of Savolainen *et al.* (36) who reported a stimulation of diacylglycerol acyltransferase activity in baboons with moderate alcoholic liver damage. The effect on this enzyme activity disappeared with progression of the liver damage, whereas serum TG levels remained elevated. Nevertheless, these authors suggested that hepatic diacylglycerol acyltransferase might be the most likely site for modulation of TG synthesis and secretion in the course of alcoholic liver damage.

It is striking that during the first 2 weeks of ethanol ingestion the amount of TG in serum VLDL and the activities of phosphatidate phosphohydrolase, diacylglycerol acyltransferase, and phosphocholine cytidylyltransferase increased (Figs. 1, 4, 5). Continuation of ethanol consumption then resulted in a slow decrease of these parameters. This may be attributed to adaptive changes developing in the liver (36, 54). It is possible that upon prolonged ethanol ingestion the ethanol-induced changes of the redox state of the liver are partly restored to normal. It was indeed demonstrated that the rate of ketogenesis in hepatocytes isolated from rats treated with ethanol for 35 days was no longer different from that of the controls, whereas the fatty acid oxidation to $CO_2$ remained lower during the entire period of ethanol feeding (14). The relative importance of each of these factors will have to be investigated.

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